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USE OF LYSOSTAPHIN TO REMOVE CELL-ADHERENT STAPHYLOCOCCI DURING *IN VITRO* ASSAYS OF PHAGOCYTE FUNCTION

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Summary.—Lysostaphin, a bacteriolytic enzyme, has been used to remove cell-adherent and extracellular *Staphylococcus aureus* from phagocyte–bacterial mixtures *in vitro*. Lysostaphin kills *S. aureus* more rapidly than penicillin, is not toxic for phagocytic cells and, when used for short periods at low concentrations, appears to enter neither human nor mouse mononuclear phagocytes. The use of lysostaphin provides the basis of a simple reliable direct *in vitro* assay for measuring the attachment and ingestion of *S. aureus* by phagocytic cells.

DIRECT *in vitro* tests of bacterial ingestion and killing by phagocytes, while simple in theory, present several technical problems. One of these is the removal of those bacteria which adhere to the surface of the phagocyte but which have not been taken up by the end of the ingestion period. Antibiotics have been used to remove adherent bacteria, but they are not wholly satisfactory for two reasons. First, antibiotics may kill bacteria too slowly to be of use in assays of short duration. Second, some antibiotics may be taken up by phagocytes and contribute to observed intracellular killing (Cole and Brostoff, 1975; Veale *et al.*, 1976).

Lysostaphin is a bacteriolytic enzyme which acts upon staphylococcal peptidoglycan (Strominger and Ghuysen, 1967). It is not taken up by polymorphonuclear leucocytes (PMN) (Tan, Watanakunakorn and Phair, 1971) and consequently has been used to remove extracellular staphylococci during assays of phagocytic function (Verhoef, Peterson and Quie, 1977). In this paper we have examined the uptake of lysostaphin by mononuclear phagocytes and its effect on ingested *S. aureus* to determine whether lysostaphin can be used in assays with these cells. We have also used lysostaphin to follow the kinetics of staphylococcal attachment to, and ingestion by, human peripheral blood phagocytes.

MATERIALS AND METHODS

Bacteria.—*S. aureus* Cowan I (NCTC 8530) and Oxford (NCTC 6571) were used with peripheral blood phagocytes and with macrophages respectively. Strain 6571 was grown overnight at 37° in 10 ml nutrient broth (Oxoid), while strain 8530 was grown under the same conditions in 10 ml Mueller Hinton broth (BBL) to which had been added 20 μ/Ci of methyl^{3}H-thymidine specific activity 5 Ci/mmol (Radiochemical Centre, Amersham). The staphylococci were centrifuged, washed twice in saline and further diluted in Medium 199.

Lysostaphin.—Lysostaphin (Becton Dickinson; Sigma) was used at a concentration of 1 mg/ml in saline. This preparation was stable for at least 4 weeks at 4°.

Preparation of phagocytic cells.—Human peripheral blood leucocytes were separated from heparinized venous blood by dextran sedimentation. Residual erythrocytes were removed by hypotonic lysis with 0·83% Tris-buffered ammonium chloride. The leucocytes were washed with Medium 199, counted and adjusted to $2{\cdot}5 \times 10^6$ polymorphonuclear leucocytes/ml in medium supplemented with autologous plasma.

Human alveolar macrophages obtained during

fibreoptic bronchoscopy were centrifuged and suspended in Medium 199. Aliquots of 10^6 cells were placed on coverslips. After incubation for 60 min at 37°, non-adherent cells were removed by washing with saline and the adherent cells cultured in fresh medium supplemented with glutamine and foetal calf serum.

Mouse peritoneal exudates were "stimulated" with proteose peptone for 72 h and obtained by peritoneal lavage with Medium 199. Mouse macrophages were prepared for assay in a similar way to the human alveolar macrophages described above. In some experiments "unstimulated" peritoneal cells were used.

Assay of peripheral blood phagocyte function.—Ingestion: following opsonization in 10% plasma medium for 30 min at 37°, mixtures of radiolabelled *S. aureus* (0·5 ml) and blood phagocytes (0·5 ml) were inoculated at 37° for 15 min on a roller. The *S. aureus*:cell ratio was 2:1. Some tubes (A) were spun for 5 min at 150 *g* to collect cells, and some (B) at 3000 *g* to collect cells and extracellular bacteria. Cell pellets A and B were washed twice with saline. The remaining tubes (C) received 20 μg lysostaphin, were incubated for a further 10 min without rolling, centrifuged at 150 *g* and washed twice with saline. After digestion with Soluene 350 (Packard) the cell pellets were counted in a liquid scintillation counter.

$$\% \text{ uptake} = \frac{\text{cpm A or C}}{\text{cpm B}} \times 100.$$

Uptake of lysostaphin by macrophages.—Uptake of lysostaphin: mouse peritoneal or human alveolar macrophages cultured on glass coverslips were allowed to ingest heat-killed *S. aureus*, washed and incubated overnight in medium with or without lysostaphin. The cells were washed, harvested, disrupted by sonication, and the cell extracts tested for their lysostaphin content by measuring their bactericidal activity against a standard inoculum of *S. aureus*.

In other experiments the killing of ingested viable *S. aureus* by these glass-adherent cells was compared over a period of 3 or 4 h in the presence of varying concentrations of extracellular lysostaphin. The cells were washed, harvested and disrupted by sonication and viable counts were made on tenfold dilutions of the sonicate.

Statistical analysis.—Student's *t* test was used throughout.

RESULTS

Killing of S. aureus *by lysostaphin*

Lysostaphin (10 μg or 20 μg) reduced the viable counts of both *S. aureus* Cowan I and Oxford by over 4 logs after 15 min incubation with the bacteria at 37° (Figs. 1 and 2). In comparison penicillin (10 μg or 50 μg) had little effect on *S. aureus* Cowan I (Fig. 1). Raising or lowering the bacterial inoculum did not affect the rate of killing by lysostaphin.

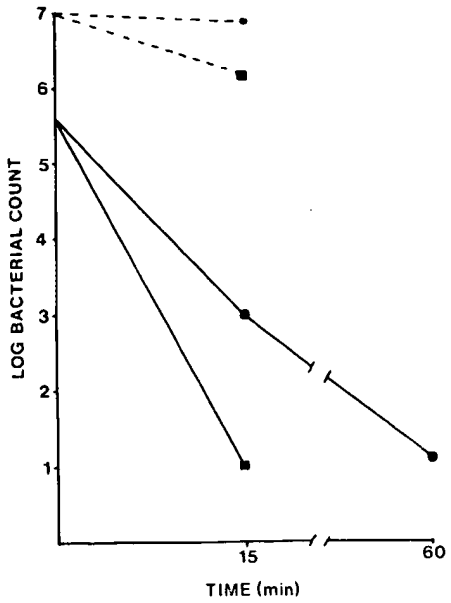
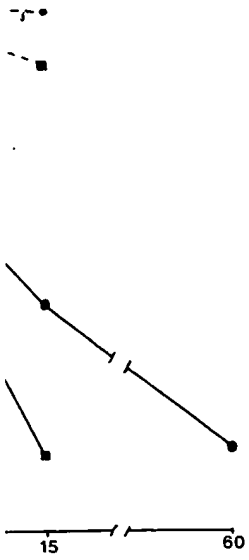


FIG. 1.—Killing of *S. aureus* Cowan 1 by lysostaphin and penicillin at 37°. ●·····● lysostaphin 2·5 μg/ml. ■ —■ lysostaphin 10 and 20 μg/ml. ● ·· ● penicillin 10 μg/ml. ■ - - ■ penicillin 50 μg/ml.

Uptake of lysostaphin by macrophages

PMN have been shown not to take up lysostaphin (Tan *et al.*, 1971), but the situation concerning mononuclear phagocytes is unclear. Cell-associated lysostaphin was detected in extracts from both human alveolar and "stimulated" mouse peritoneal macrophages which had been cultured overnight in the presence of enzyme following the ingestion of heat-killed *S. aureus* (Table I). Extracts of macrophages incubated without lysostaphin had no bactericidal activity.

The survival of ingested *S. aureus* within human alveolar or "stimulated" mouse peritoneal macrophages was significantly reduced when these cells were incubated for several hours in the presence of increasing concentrations of extracellular



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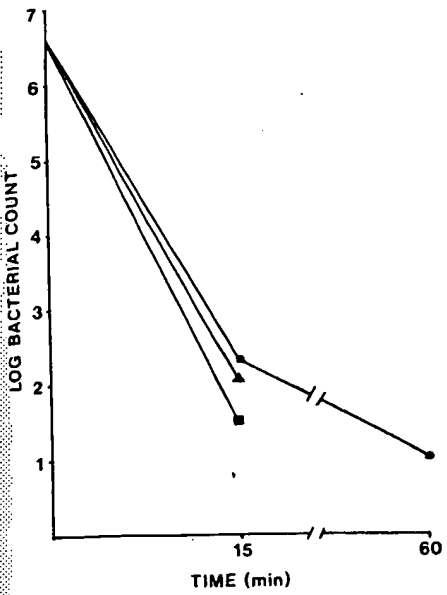

FIG. 2.—Killing of *S. aureus* Oxford by lysostaphin at 37°. ●——● lysostaphin 2·5 μg/ml. ▲——▲ lysostaphin 10 μg/ml. ■——■ lysostaphin 20 μg/ml.

lysostaphin (Table II). However, this effect was not seen with "unstimulated" mouse cells (Table III). At concentrations of lysostaphin below 5 μg/ml there was no further reduction of viable intracellular *S. aureus*. We have been unable to obtain sufficient human alveolar macrophages to investigate this low dose range. No viable extracellular *S. aureus* was detected in lysostaphin supernatants.

Attachment and ingestion of S. aureus *by blood phagocytes*

After 15 min incubation 86% of the

TABLE I.—*Measurement of Lysostaphin in Macrophage of Extracts by Titration against live* S. aureus

Macrophage extract	Exposure of macrophages to lysostaphin	Growth of *S. aureus* Dilution of extract 1/1	1/4	1/10
Human	Yes	—	ND	+
Human	No	+	ND	+
Mouse	Yes	—	—	ND
Mouse	No	+	ND	ND

+ = growth of *S. aureus*
— = no growth

TABLE II.—*Survival of* S. aureus *within Human and "Stimulated" Mouse Macrophages after Exposure to Extracellular Lysostaphin for 3 h (Human) and 4 h (Mouse)*

Lysostaphin concentration (μg/ml)	Viable intracellular *S. aureus* (c.f.u. ±SD) Mouse cells	Human cells
50	29±4	10(5±2)*
25	41±12	ND
12·5	ND	45±15
5	55±10	ND
4	41±5	ND
3	56±9	ND
2·5	ND	76±21
2	44±4	ND

* Actual colony count.

radiolabelled bacterial inoculum was cell-associated (not removed by washing), 32% being removed by treatment with lysostaphin (Table IV). This 32% probably represented *S. aureus* attached to phagocyte surfaces but not ingested. The remaining 54%, which was resistant to lysostaphin, represented intracellular *S. aureus*. The use of heat-inactivated plasma (56° for 30 min) reduced the number of intracellular *S. aureus* (lysostaphin resistant) by 40% ($P=<0·01$), but only lowered the total number of cell-associated staphylococci by 23%. Heat-labile opsonins appear in this system to be more involved with bacterial ingestion than with their attachment to phagocytes.

The attachment and subsequent ingestion of *S. aureus* by phagocytes is a dynamic process which we have been able to demonstrate using lysostaphin. Between

TABLE III.—*Survival of* S. aureus *within "Unstimulated" Mouse Macrophages after Exposure to Extracellular Lysostaphin for 4 h*

Lysostaphin concentration (μg/ml)	Time (hr)	Viable intracellular *S. aureus* (c.f.u. ×10⁻⁵) Experiment 1	2	3
—	0	69	74	144
5	3	7·4	7·5	15·0
10	3	6·6	11·5	13·0
20	3	6·7	7·0	15·7
40	3	5·8	7·8	12·5

TABLE IV.—*Uptake of Radiolabelled* S. aureus *by Human Blood Macrophages*

Source of opsonin	Mean % of cell-associated *S. aureus* (±SD) With lysostaphin	Without lysostaphin
Normal plasma	54±12	86±14
Heated plasma	14±4	63±11

15 and 30 min there was a fall in the number of adherent *S. aureus* and a corresponding rise in the percentage of intracellular bacteria (Table V). The minimum

DISCUSSION

The ideal agent for removing cell-adherent and extracellular bacteria from phagocyte-bacterial mixtures *in vitro* should, under assay conditions:

1. Kill bacteria rapidly.
2. Not be toxic for phagocytes.
3. Not enter or be taken up by phagocytes.
4. Be active against a wide range of bacteria.

Lysostaphin kills staphylococci rapidly without being cytotoxic and this enzyme does not appear to be taken up by human PMN (Tan *et al.*, 1971). The macrophage differs from the PMN in that it appears to be more actively pinocytic and therefore more likely to take up soluble materials from its environment. Furthermore, unlike the PMN, the macrophage can be "activated" by a variety of stimuli to increase its metabolic and functional activity. There have not been any previous reports on the behaviour of human macrophages following exposure to lysostaphin but, like Baughn and Bonventre (1975), we found no evidence of intracellular lysostaphin following its use to remove cell-adherent *S. aureus* from "unstimulated" mouse peritoneal macrophages. Our results indicate that lysostaphin, when used in sufficient concentration for sufficient time, is taken up by "stimulated" mouse peritoneal macrophages and lavaged human alveolar macrophages. These findings, together with those of Baughn and Bonventre (1975), show the effect of using "stimulated" rather than "unstimulated" mouse peritoneal macrophages. With mouse cells we found no increase in viable *S.*

TABLE V.—*Uptake of* S. aureus—*Changes in Adherent and Intracellular Bacterial Populations with Time*

Experiment	% Intracellular *S. aureus* (lysostaphin-resistant) T_{15}	T_{30}	% Adherent *S. aureus* T_{15}	T_{30}	% Change in bacterial population between T_{15} and T_{30} Intracellular	Adherent
1	63	86	31	9	+23	−22
2	65	81	15	5	+16	−10

TABLE VI.—*Uptake of* S. aureus—*Effect of Plasma Concentration*

% Plasma concentration	% Uptake of *S. aureus* 8530 Experiment 1	2	3	Mean
0	2	4	5	4
2·5	10	9	8	9
5·0	21	19	19	20
10·0	52	66	53	57
20·0	55	58	61	58

concentration of autologous plasma required for optimum uptake of *S. aureus* was 10% (Table VI). In recent experiments we have reduced the concentration of lysostaphin from 20 μg/ml to 10 μg/ml without affecting our results.

We compared the ingestion of *S. aureus* measured by radioactive counts with that measured by viable counts. The mean uptake was 20% with the latter method as compared with 54% with the former.

Radioactive counting measures dead as well as live bacteria. The lower figures obtained with viable counts is probably the result of the killing of ingested bacteria during the initial incubation period.

aureus when we reduced the lysostaphin concentration below 5 μg/ml. As a result of the low intracellular bacterial counts involved and the risk of extracellular bacterial proliferation at very low lysostaphin concentrations, we cannot definitely state that there was no cellular uptake of lysostaphin at levels below 5 μg/ml. There is, however, a wide safety margin between the conditions (concentration and exposure time) needed for the removal of extracellular and cell-adherent *S. aureus* by lysostaphin and those under which detectable amounts of this enzyme are taken up by macrophages.

Lysostaphin can only be used with staphylococci as it acts upon amino acid cross-linkages peculiar to staphylococcal cell wall peptidoglycan (Strominger and Ghuysen, 1967). There are, however, other bacteriolytic enzymes which have a wider range of activity (Strominger and Ghuysen, 1967). It may be possible to use these in a similar way to lysostaphin to study the phagocytosis of other bacteria, especially Gram-negative organisms.

Lysostaphin can be used to measure the cell-adherent bacterial population as well as simply to remove contaminating extracellular bacteria. After 15 min incubation 15–30% of the staphylococcal inoculum was cell-adherent. This is higher than the 10% reported by Verhoef *et al.*, (1977) but this may be the result of differences in technique and in their use of serum rather than plasma as a source of opsonin. After 30 min incubation the cell-adherent staphylococcal population had fallen to below 10% with a corresponding rise in the percentage of intracellular bacteria. It may be of value, in patients with defects in bacterial ingestion, to study the change with time of the adherent and intracellular bacterial populations.

Assays based on the removal of extracellular *S. aureus* with lysostaphin have been used to demonstrate phagocytic defects in cells from patients with chronic granulomatous disease (Verhoef *et al.*, 1977; Biggar, 1975) and diabetes mellitus (Tan *et al.*, 1975). In addition to these clinical applications we are using lysostaphin to study the penetration of antimicrobial drugs into actively phagocytic cells.

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Requests for reprints should be addressed to C. S. F. Easmon, Bacteriology Department, Wright–Fleming Institute, St Mary's Hospital Medical School, London, W2 1PG.

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